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Differential Expression of Drug Resistance Genes and Chemosensitivity in Glial Lineages Correlate with Differential Response of Oligodendrogliomas and Astrocytomas to Chemotherapy¹

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ABSTRACT

The two principal subtypes of glial neoplasms, astrocytomas and oligodendrogliomas, exhibit striking differences in response to chemotherapy. This differential chemosensitivity might be explained by the specific genetic alterations causing gliomas but could also be attributable to specific properties intrinsic to the cells from which gliomas arise. To examine the possibility that chemosensitivity might be associated with lineage-specific properties of potential ancestors of these tumors, we explored: (a) the expression of drug resistance genes in rat glial cells; (b) the sensitivity of rat glial subtypes to the bifunctional alkylating agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); and (c) the effect of *O*⁶-methylguanine-DNA methyltransferase (MGMT) and glutathione modulation on resistance to BCNU. Astrocytes, O-2A progenitors, and oligodendrocytes each displayed a unique pattern of expression of six drug resistance genes: *MGMT*, *GST* μ , *GST* π , *p53*, *MDR*, and *MT*. Oligodendrocytes were more sensitive to BCNU than either astrocytes or O-2A progenitors. The increased resistance of astrocytes in comparison to oligodendrocytes was modulated, at least in part, by both *O*⁶-benzylguanine (BG) and DL-buthionine-(S,R)-sulfoximine, suggesting a role for both MGMT and glutathione in the resistance of astrocytes to BCNU. The sensitivity of O-2A progenitors to BCNU following BG pretreatment is virtually indistinguishable from that of oligodendrocytes depleted of MGMT, suggesting that the down-regulation of MGMT is sufficient to account for the increased sensitivity of oligodendrocyte lineage cells to BCNU as they differentiate. These experiments provide support for the hypothesis that properties of glial cells retained in gliomas may contribute to the differential chemosensitivity of glial neoplasms.

INTRODUCTION

The most common primary brain tumors in humans are the gliomas. There are two principal subtypes of glial neoplasms, each with strikingly different chemosensitivities. Astrocytomas are relatively resistant to DNA alkylating drugs (e.g., nitrosoureas), whereas 70% of oligodendrogliomas, including anaplastic (malignant) oligodendrogliomas, are exquisitely sensitive to the nitrosourea-based chemotherapy regimen, procarbazine/CCNU⁴/vincristine (1-7). Because the outcome of treatment for these two types of glioma is so different, it is

of considerable importance to understand what aspects of the clinical history of these tumors might account for this differential sensitivity.

There are two very different possible explanations for the variation of chemosensitivity or chemoresistance in tumors. One explanation, and the one that currently takes precedence in analyzing this problem, is that these differences are attributable to altered gene expression patterns associated with the process of neoplastic transformation. According to this hypothesis, it would be expected that certain tumors acquire a chemoresistant profile as a result of aberrant gene expression that protects against cell death. Examples of such genes as *bcl-2*, multidrug resistance genes, certain members of the glutathione-S-transferase family, and an increasing number of other genes are associated with the expression of a chemoresistant phenotype (8-10). As yet, there is no direct evidence that genetic alterations causing glial tumors are also chemosensitizing alterations, but recently Cairncross *et al.* (11) reported that allelic loss of chromosome 1p, an early molecular event in the genesis of oligodendrogliomas, is significantly associated with sensitivity to procarbazine/CCNU/vincristine chemotherapy.

Another possible explanation for the expression of chemosensitivity or chemoresistance in tumors is that these differences are related to the biology of the specific cells of origin. Such a possibility is of particular interest in respect to the differential response of astrocytomas and oligodendrogliomas to chemotherapy because of the observations that, at least in rats, tumors of the oligodendroglial lineage are preferentially induced after transplacental exposure to ethylnitrosourea (12, 13). This result is consistent with the possibility that normal cells that have been thought to give rise to oligodendrogliomas may be especially vulnerable to the effects of this particular nitrosourea.

Sensitivity to nitrosoureas (BCNU, CCNU, and methyl-CCNU being the most common) is of particular interest in respect to chemotherapy, because these lipid-soluble drugs are capable of penetrating into the central nervous system for treatment of intracranial tumors. Of these, BCNU has been the most frequently studied chemotherapeutic agent in the treatment of glial tumors (14-16). Many mechanisms of resistance have been implicated for BCNU. One of the best characterized mechanisms is the DNA repair protein MGMT (17-20). MGMT repairs DNA damage by removing alkyl groups from the *O*⁶ position of guanine, a critical site of alkylation by the nitrosoureas. In glial cells, resistance to BCNU and other alkylating agents can be mediated by MGMT, and depletion of MGMT activity by BG has been shown to reverse resistance (21-25).

A second mechanism of resistance to the nitrosoureas that has received significant attention is the detoxification pathway of glutathione and its associated GSTs. The tripeptide glutathione plays a role in detoxification by nonenzymatic conjugation of alkylating agents and other electrophilic molecules with its thiolate moiety (26). Conjugation of drug with glutathione results in a more water-soluble and less toxic product (27). GSTs are a family of enzymes that catalyze the conjugation of these drugs, thereby enhancing the

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⁴ The abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; anti-RAN-2, anti-rat neural antigen-2; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; bFGF, basic fibroblast growth factor; BG, *O*⁶-benzylguanine; B-S, Bottenstein-Sato; BSO, DL-buthionine-(S,R)-sulfoximine; FBS, fetal bovine serum; GC, galactocerebroside; GFAP, glial fibrillary acidic protein; GST, glutathione-S-transferase; MDR, multidrug resistance; MGMT, *O*⁶-methylguanine-DNA methyltransferase; MT, metallothionein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; O-2A, oligodendrocyte-type-2 astrocyte; PDGF, platelet-derived growth factor.

detoxification. GST isoenzymes are broadly grouped into three classes (28), and each isoenzyme class exhibits different but potentially overlapping substrate specificities. Glutathione and GST levels have been studied in human glioma tissues and cell lines, and support for a role of these pathways in resistance to alkylating agents has been provided (29-31). In addition, modulation of glutathione has been demonstrated to alter resistance to a number of alkylating agents, including BCNU (29, 30, 32-35).

In the present study, we explored: (a) the expression of drug resistance genes in cultures of purified rat glial cells; (b) the sensitivity of rat astrocytes, O-2A progenitors, and oligodendrocytes to the bifunctional alkylating agent, BCNU; and (c) the effect of MGMT and glutathione modulation on resistance to BCNU in purified rat glial cells. We have found that there is an excellent correlation between the chemosensitivity of glial cells and the tumors to which they are thought to be related. In addition, the mechanisms that seem to be responsible for the differential response to BCNU also show an excellent correlation with mechanisms that are thought to play a role in the differing chemosensitivities of astrocytomas and oligodendrogliomas.

MATERIALS AND METHODS

Materials. DMEM, L15 medium, HBSS, FBS, antibiotics, and growth factors were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada). All other cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO). BCNU was obtained from Bristol Laboratories of Canada (Montreal, Quebec, Canada). O⁶-Benzylguanine was kindly provided by Dr. Robert C. Moschel of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The anti-RAN-2 (36) and A2B5 antibody (37) hybridomas were purchased from the American Type Culture Collection (Rockville, MD). Anti-GC hybridoma cells (38) were raised previously by Dr. Mark Noble. Anti-GFAP antibody, rhodamine- and fluorescein-conjugated secondary antibodies, biotinylated antirabbit IgG, and 7-amino-4-methylcoumarin-3-acetic acid streptavidin were purchased from Dimension Laboratories, Inc. (Mississauga, Ontario, Canada). Radiolabeled nucleotides [³²P]dCTP and [³²P]dATP were purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada). All molecular biology grade chemicals were purchased from BDH, Inc. (Toronto, Ontario, Canada), Fisher Scientific (Ottawa, Ontario, Canada), Life Technologies, and Sigma Chemical Co.

Isolation of Astrocyte Cultures. Astrocyte cultures were isolated from newborn (1-3 days of age) rat cerebrums using a method modified from Carthy and De Vellis (39). Cerebral hemispheres were isolated from unanesthetized Sprague Dawley neonates and dissected free of meninges and blood vessels. The tissue was minced and treated with 0.025% trypsin for 30 min at 37°C. Trypsin inhibitor with 40 µg/ml DNase was added and incubated 5 min to stop the digestion of the tissue. The tissue was centrifuged in a Beckman Model TJ-6 centrifuge (Beckman Instruments, Inc., Fullerton, CA) at 1000 rpm, the pellet was resuspended in 40 µg/ml DNase and centrifuged in a 10-ml pipette to dissociate cells. The resultant single cell suspension was centrifuged at 1000 rpm for 5 min, resuspended in DMEM supplemented with 10% FBS and 50 units/ml penicillin/streptomycin, and seeded into polylysine-coated, 80-cm² flasks (2 × 10⁷ cells/flask). To remove contaminating cells, cultures were harvested after 48 h with 0.05% trypsin and 2 mM EDTA and replated. Cells were grown on coverslips for various time intervals under the above-mentioned conditions before being used for immunocytochemical analysis.

Isolation of Purified O-2A Progenitors and Oligodendrocytes. Populations of purified O-2A progenitors and oligodendrocytes were isolated from 10-day-old rat corpus callosa using an immunopanning purification method described by Mayer *et al.* (40). Panning plates were prepared 1 day in advance by incubating 10 ml of Tris buffer solution (50 mM, pH 9.5) with 50 µg/ml anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA) in a 96-well dish overnight at 4°C. The next day, each dish was washed three times with PBS and incubated with anti-RAN-2 hybridoma supernatant (1:4) in PBS containing 0.8 mg/ml BSA for 1 h at 37°C. The addition of BSA was to block the nonspecific adherence of cells to the panning plates. The

dishes were then washed three times with PBS, and PBS was left on the dishes until they were required for panning. Twelve anti-RAN-2 plates were prepared for each litter of 12-18 rats.

Corpus callosa were isolated from six to nine Sprague Dawley neonates, minced finely, and incubated in 750 µl of 667 units/ml solution of collagenase in L15 medium for 45 min at 37°C. The tissue was then centrifuged at 1000 rpm in a Beckman Model TJ-6 centrifuge for 5 min and resuspended in 3 ml of a 30 unit/ml papain solution in DMEM containing L-cysteine and 0.013% DNase. This suspension was incubated for 1 h at 37°C, centrifuged, and resuspended in 1 ml of papain inhibitor solution consisting of 2 mg/ml ovomucoid and 1 mg/ml BSA in L15 medium. The tissue was then triturated sequentially through 23- and 27-gauge needles to yield a single-cell suspension. Two of these preps were performed in parallel for each litter of 12-18 rats. The corpus callosa cell suspension was resuspended in B-S medium (41) as modified by Lillien *et al.* (42). This defined medium consisted of 5.6 mg/ml glucose, 5 µg/ml insulin, 100 µg/ml transferrin, 100 µg/ml BSA, 0.06 ng/ml progesterone, 16 µg/ml putrescine, 40 ng/ml selenium, 40 ng/ml thyroxine, and 30 ng/ml tri-iodothyronine in DMEM.

To deplete astrocytes and meningeal cells, the suspension was divided evenly into six samples and incubated on anti-RAN-2 panning plates for 30 min at 37°C. This incubation also depletes microglia and macrophages that stick via their Fc receptors to the original IgG coating on the panning plate. The plates were agitated gently at 15 min to ensure access of the panning surface to all cells. The nonadherent cells were transferred to a second set of six anti-RAN-2 plates for an additional 30-min incubation. All cells remaining in the supernatant were seeded into polylysine-coated, 80-cm² flasks in B-S medium containing 0.5% FBS and 10 ng/ml each of PDGF-AA and bFGF.

O-2A progenitor cells were grown in the presence of 10 ng/ml each of PDGF-AA and bFGF to promote cell proliferation and prevent differentiation. To obtain pure populations of oligodendrocytes, O-2A progenitors were first grown to the required cell number. O-2A progenitors were then allowed to undergo differentiation into oligodendrocytes by removing bFGF from the medium. All medium was removed from the flasks, and the progenitors were washed once with citrate saline buffer (15 mM sodium citrate and 130 mM KCl). Fresh B-S medium containing 0.5% FBS and 10 ng/ml PDGF-AA was added to the flasks, and the cells were allowed to differentiate for ~4 days. The state of differentiation was monitored morphologically; oligodendrocytes displayed highly branched processes in contrast to the bipolar morphology displayed by O-2A progenitors. That such cultures consisted of >90% oligodendrocytes was confirmed by staining with cell type-specific antibodies. Because the oligodendrocytes began to undergo cell death within several days after reaching terminal differentiation, all experiments were completed within 1 week of the original change in morphology. In all cases, the requisite growth factors were replenished every 24 h. Both cell types were grown on coverslips for various time intervals under the above-mentioned conditions before being used for immunocytochemical analysis.

Immunocytochemistry. Anti-GFAP antibody (rabbit IgG), A2B5 antibody (mouse IgM), and anti-GC antibody (mouse IgG3) were used to ascertain the percentage of astrocytes (GFAP-positive, A2B5-negative, and GC-negative), O-2A progenitor cells (A2B5-positive, GC-negative, and GFAP-negative), and oligodendrocytes (GC-positive, A2B5-negative, and GFAP-negative) in the pure cultures using a triple staining protocol. All dilutions and washes were carried out using Hank's staining solution [HBSS without sodium bicarbonate, 5% calf serum, 4.76 g/l HEPES (M, 238.3), 5.2 g/l HEPES (M, 260.3), and 0.05% sodium azide]. Briefly, coverslips containing cells were washed once by dipping the coverslip in Hank's staining solution, and cells were fixed with 4% paraformaldehyde for 15-20 min. After all subsequent incubations, the coverslips were washed by dipping in four changes of Hank's staining solution. A mixture of 1/2 GC-1/2 A2B5 hybridoma supernatants was added for 20 min and washed off, and cells then were fixed in methanol for 15 min at -20°C to permeabilize the cell membranes. A mixture of anti-GFAP (1:300), antimouse IgG3-fluorescein (1:100), and antimouse IgM-rhodamine (1:100) was then added to the cells. Goat antirabbit biotin (1:100) was added for 20 min, followed by 7-amino-4-methylcoumarin-3-acetic acid streptavidin (1:50) for 40 min. After the final wash, coverslips were rinsed in distilled water, mounted onto slides with a drop of antifade [2.5% 1,4-diazabicyclo-(2.2.2) octane in glycerol], and sealed with nail polish. Cells were visualized with fluorescence optics on a Leitz Diavert inverted microscope (Ernst Leitz Canada, Ltd.,

Midland, Ontario, Canada), and culture purity was determined by counting the percentage of positive cells.

Northern Blot Analysis. Sequences for oligonucleotide probes are as follows: *MGMT*, 5'-ttcttaccg gcaattagca gccctggcag gcaaccccaa agc-3'; *GST* μ , 5'-cggcagaat ccgctctcc tctgtctc caccagtg-3'; and *GST* π , 5'-ggtaac-cacc tctctctcc agctctggcc ctgtcagcc-3'.

All cDNA inserts used for Northern hybridization were generously donated by other investigators. A 1.35-kb mouse *p53* partial cDNA in pUC-18, designated pMO53, was a gift from Dr. Sam Benchimol (Ontario Cancer Institute, Toronto, Ontario, Canada). A derivative of pMO53, pECM53, has been described by Johnson *et al.* (43). The hamster *MDR* cDNA in pUC-9, designated pEX/172 (44), and the primers (A100 and B100) required to label the probe using a PCR protocol were kindly provided by Dr. Victor Ling (British Columbia Cancer Research Center, Vancouver, British Columbia, Canada). The *MT* cDNA was generously provided by Dr. Jim Koropatnick (London Regional Cancer Center, London, Ontario, Canada).

Total RNA was isolated from cultured cells using TRIzol reagent (Life Technologies, Inc., Burlington, Ontario, Canada), following the manufacturer's instructions. Selection of mRNA was performed using Oligo(dT) Cellulose Columns (Life Technologies). As described (45), samples and molecular weight markers (0.24–9.5 kb; Life Technologies) were electrophoresed at 50 mA for 3–4 h in 1.1% agarose gels containing formaldehyde; mRNA then was transferred to Gene Screen Plus membranes (DuPont Canada, Mississauga, Ontario, Canada) in 10 \times SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) by capillary action.

To block nonspecific binding, membranes were prehybridized in a VWR Scientific Model 2710 hybridization oven (Toronto, Ontario, Canada) at 42°C in a buffer containing 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl and 100 μ g/ml herring sperm DNA. After 1–4 h, radiolabeled probe (10⁶ cpm/ml) was added to the prehybridization solution and incubated with constant rotation for 16–20 h at 42°C. 5'-Labeling of oligonucleotide probes was carried out with T4 polynucleotide kinase, and labeling of cDNA probes was performed using the Life Technologies Random Primers DNA Labeling System, following the manufacturer's instructions (Life Technologies). A PCR-based method was used to label the pEX/172 (*MDR*) cDNA probe (44). Subsequently, probe-specific washing procedures were followed.

Membranes were exposed to a Storage Phosphor Screen (Molecular Dynamics, Inc., Sunnyvale, CA), screens were scanned by a Personal Densitometer SI Model 375A (Molecular Dynamics, Inc.), and images were quantitated using ImageQuant software (Molecular Dynamics, Inc.). All images were below saturation and corrected for background. Expression of standard control probes, β -actin and glyceraldehyde-3-phosphate dehydrogenase, varied slightly with the degree of differentiation of oligodendrocyte lineage cells (data not shown); as such, quantified signal intensities for each probe were normalized to a poly-dT probe. Images were assembled into composites using CorelDraw software (Corel Corp., Ottawa, Ontario, Canada), and signal intensity data were analyzed using Microsoft Excel software (Microsoft Canada, Inc., Mississauga, Ontario, Canada).

Cytotoxicity Analysis. BCNU was dissolved in absolute alcohol and diluted to 3.3 mg/ml with sterile water. The stock solution was stored at –80°C, and subsequent dilutions were made with culture medium at the time of cell treatment. BG stock solution (100 mM) was made in filter-sterilized DMSO and stored at –80°C. At the time of use, BG was diluted to 1 mM in PBS, and subsequent dilutions were made with culture medium. Twenty-five μ M BSO was prepared in medium at the time of treatment.

To test for cytotoxicity, 96-well plates were seeded at 10⁴ cells/well and incubated at 37°C. Forty-eight h later, cells were treated and incubated for an additional 96 h, at which time cytotoxicity was measured using the MTT assay. BCNU treatments were performed for 2 h. On the basis of previous reports, pretreatment with modulators consisted of a 2-h incubation for BG (46) and a 24-h incubation for BSO (47, 48) to modulate *MGMT* and glutathione, respectively.

The toxic effects of the above treatments were compared using the MTT assay as modified for chemosensitivity testing as described by Cole (49). Ninety-six h after treatment, 100 μ l of medium were removed, 25 μ l of MTT solution (2 mg/ml in PBS) were added, and the plates were incubated for 2 h at 37°C. To solubilize formazan crystals, 0.04 M hydrochloric acid in isopropanol was added to each well and mixed thoroughly. Plates were kept at 37°C for 1 h, and cell viability was quantified by measuring light absorbance (570

nm) in a Bio-Rad Model 3550 automated microplate reader (Bio-Rad Laboratories, Richmond, CA). To control for differential growth rates, response curves were normalized by expressing absorbance values relative to nontreated control values. Cytotoxicity assay results were analyzed using a two-tailed *t* test for independent means.

RESULTS

Characterization of Cultures of Purified Rat Glial Cells. The cellular composition of the cultures was determined at the time of assay by immunocytochemical analysis with cell type-specific antibodies. Astrocyte cultures (GFAP-positive, A2B5-negative, and GFAP-negative) were >95% pure. O-2A progenitors (A2B5-positive, GFAP-negative, and GC-negative) were cultured to a purity of >90% and oligodendrocytes (GC-positive, A2B5-negative, and GFAP-negative) to a purity >90%.

Genes Associated with Chemoresistance Are Expressed at Different Levels in Different Glial Cell Populations. To explore expression of drug resistance genes in cultures of purified glial cells, RNA was isolated from astrocytes, O-2A progenitor cells, and oligodendrocytes, poly(A) selected, and subjected to Northern blot analysis. The Northern blots were probed for *MGMT*, *GST* μ , *GST* π , *p53*, *MDR*, and *MT* transcripts (Fig. 1). Hybridization signals were normalized to a poly-dT probe (data not shown). A differential pattern of mRNA expression was observed for each of the six probes. In astrocytes, expression levels of *MGMT*, *p53*, and *MDR* mRNA were high; astrocytes displayed a 5-fold increase in both *MGMT* and *MDR* expression over oligodendrocytes; and an approximate 2-fold increase was exhibited in *p53* expression. In comparison, expression



Fig. 1. Differential expression of drug resistance genes in cultures of purified glial cells. RNA was isolated from astrocytes, O-2A progenitor cells, and oligodendrocytes, poly(A) selected, and subjected to Northern blot analysis. A differential pattern of mRNA expression was observed for each of the six probes.

levels of *GST* μ and *MT* mRNAs were slightly higher in oligodendrocytes than in astrocytes.

Within the oligodendroglial lineage, we observed changes in expression levels of several genes as O-2A progenitor cells differentiated into oligodendrocytes. Progenitor cells displayed higher levels of *MGMT*, *GST* π , *p53*, and *MDR* expression as compared with oligodendrocytes (Fig. 1). Progenitors displayed an ~5-fold increase in *MGMT* expression over oligodendrocytes, *GST* π and *p53* expression in progenitors was approximately twice that of oligodendrocytes, and the ~3.5-fold higher level of *MDR* expression was displayed over oligodendrocytes. In contrast, oligodendrocytes displayed a higher level of expression of *GST* μ and *MT* mRNAs; the expression of *GST* μ was approximately twice that seen in O-2A progenitor cells, and *MT* mRNA levels were slightly higher in oligodendrocytes than in the progenitor cells from which they were derived.

Two mRNA transcripts were detected for each of *p53* and *MDR*. Although two transcripts have not been demonstrated previously in rat tissue, alternatively spliced forms of *p53* have been documented in mouse tissues (50, 51). Two transcripts have been detected previously for *MDR* in rat and are known to correspond to the *MDR1a* and *MDR1b* genes (larger and smaller transcripts respectively; Ref. 52).

Oligodendrocytes Are More Sensitive to Killing by BCNU Than Are O-2A Progenitor Cells or Astrocytes. To explore the potential functional consequences of differential expression of drug resistance genes in glial cells, we next examined the effect of BCNU treatment on the viability of purified rat glial cells. It had been observed previously that mixed cultures of rat cells from the oligodendrocyte lineage were more sensitive to the bifunctional alkylating agent BCNU than astrocytes (53). To expand upon this finding, we determined the sensitivity of purified rat astrocytes, O-2A progenitor cells, and oligodendrocytes to treatment with BCNU. Cells were isolated from newborn rats and treated with increasing concentrations of BCNU and cell viability was determined using the MTT assay. Oligodendrocytes were more sensitive to BCNU than either astrocytes or O-2A progenitor cells (Fig. 2). Astrocytes appeared somewhat more sensitive to BCNU than O-2A progenitors, but this difference did not reach statistical significance.

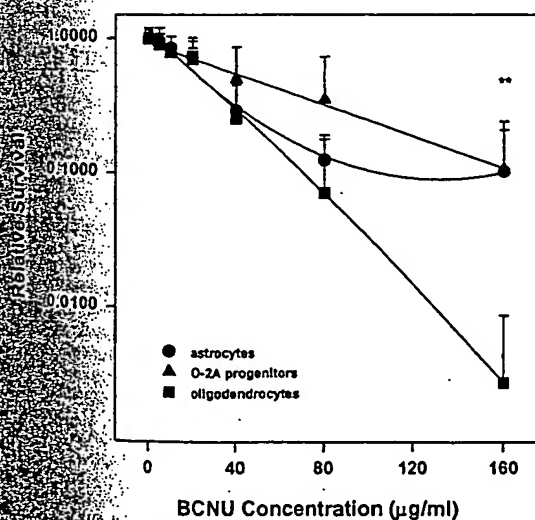


Fig. 2. Effect of BCNU treatment on purified rat astrocytes, O-2A progenitor cells, and oligodendrocytes. Both astrocytes and O-2A progenitor cells displayed greater resistance to BCNU than oligodendrocytes. O-2A progenitor cells exhibited a trend toward greater resistance to BCNU when compared with astrocytes, but this difference did not reach statistical significance. The survival of untreated cells was normalized to a value of 1.0. Each point represents the mean of at least three independent experiments, each performed in triplicate. ●, astrocytes; ▲, O-2A progenitors; ■, oligodendrocytes. Bars, SE; **, statistical significance (*, $P < 0.005$).

Because oligodendrocytes and O-2A progenitor cells are grown in chemically defined medium, we determined whether growth in such medium might account for the differential sensitivity of oligodendrocytes to BCNU. When astrocytes were grown in the same medium used for growth of oligodendrocyte lineage cells, no difference was seen in their response to BCNU (data not shown).

Sensitivity of Astrocytes to BCNU Can Be Induced by Depletion of MGMT Activity or by Inhibition of Glutathione Synthesis. There may be many reasons for resistance of astrocytes to BCNU. On the basis of previous studies, however, levels of MGMT activity and levels of glutathione were of particular interest. Rat astrocyte cultures have been found previously to have 3-fold higher levels of MGMT activity than mixed cultures of oligodendrocyte lineage cells (53). Moreover, modulation of glutathione levels has been demonstrated to alter resistance to a number of alkylating agents, including BCNU (29, 30, 32–35). In this regard, it is potentially relevant that Thorburne and Juurlink (54) demonstrated that astrocytes had 3-fold higher glutathione concentrations than O-2A progenitor cells.

Analysis of the effects of BG and BSO on BCNU sensitivity in astrocytes indicates that depleting MGMT activity or inhibiting glutathione production both increase sensitivity to this alkylating agent. Cells were pretreated with 25 μ M BG (to deplete MGMT activity) for 2 h and exposed to increasing concentrations of BCNU, and cell viability was determined using the MTT assay. As shown in Fig. 3A, such treatment increased significantly the sensitivity of astrocytes to BCNU. Similar results were obtained by treating astrocytes with BSO to inhibit glutathione synthesis. As shown in Fig. 3B, a 24-h pretreatment with 25 μ M BSO was also associated with a significant increase in sensitivity of these cells to treatment with BCNU.

Effect of BG Pretreatment on the Sensitivity of Purified O-2A Progenitors and Oligodendrocytes to BCNU. In light of previous reports that O-2A progenitor cells have low glutathione levels as compared with astrocytes (54) and our own finding that MGMT activity is higher in O-2A progenitor cells than in oligodendrocytes, we determined whether depletion of MGMT activity with BG might render O-2A progenitor cells as sensitive as oligodendrocytes to BCNU. We found that pretreatment of O-2A progenitor cells with 25 μ M BG for 2 h was sufficient to significantly increase the sensitivity of these cells to BCNU (Fig. 4). BG pretreatment also somewhat increased the sensitivity of oligodendrocytes to BCNU, although to a lesser extent than was seen with O-2A progenitor cells (Fig. 4, A and 4B). Interestingly, after negating the effects of MGMT on drug resistance by pretreatment with BG, O-2A progenitors and oligodendrocytes had similar sensitivities to BCNU (Fig. 4C).

DISCUSSION

In this study, we have documented differential expression of drug resistance genes in astrocytes, O-2A progenitor cells, and oligodendrocytes. These findings lend credence to the hypothesis that properties intrinsic to the cells from which gliomas arise, if retained, may contribute to the differential chemosensitivities of glial neoplasms.

The lineage origin of oligodendrogliomas has been a topic of interest for many years and remains uncertain. Previous studies have suggested that oligodendrogliomas may express some properties of O-2A lineage cells (55). More recent evidence that tumors of this classification express NG2 proteoglycan and the PDGF- α receptor (which are expressed by O-2A progenitor cells) lends support to the view that, as implied by their name, oligodendrogliomas may indeed be related to the oligodendrocyte lineage (56, 57). Thus, it is becoming clearer that consideration of the properties of cells of the O-2A lineage may provide information of relevance to understanding the properties of oligodendrogliomas.

A differential pattern of mRNA expression was observed for each of the six drug resistance genes that we examined: *MGMT*, *GST* μ , *GST* π , *p53*, *MDR*, and *MT*. Rat astrocytes expressed higher levels of *MGMT*, *p53*, and *MDR* than oligodendrocytes. The results for *MGMT* are consistent with our previous observations of increased *MGMT* activity in rat astrocytes relative to mixed cultures of oligodendrocyte lineage cells (53).

One of the best-studied mechanisms of resistance to BCNU is *MGMT* (17–20). *MGMT* levels show a striking correlation with responsiveness to BCNU in patient populations (58). In addition, it has been found that levels of *MGMT* in oligodendrogliomas and mixed oligo-astrocytomas are significantly lower than in astroglial tumors (59). Because *MGMT* protein activity has been found to correlate with resistance to BCNU in cultures of mixed glial cells (53), the effect of pretreatment with the modulator, BG, on the sensitivity of astrocytes to BCNU was determined. BG pretreatment significantly increased the sensitivity of astrocytes to BCNU, consistent with the hypothesis that this protein is important in conferring resistance to these cells. It was interesting to see, however, that astrocytes treated with BG nevertheless remained more resistant to BCNU than un-

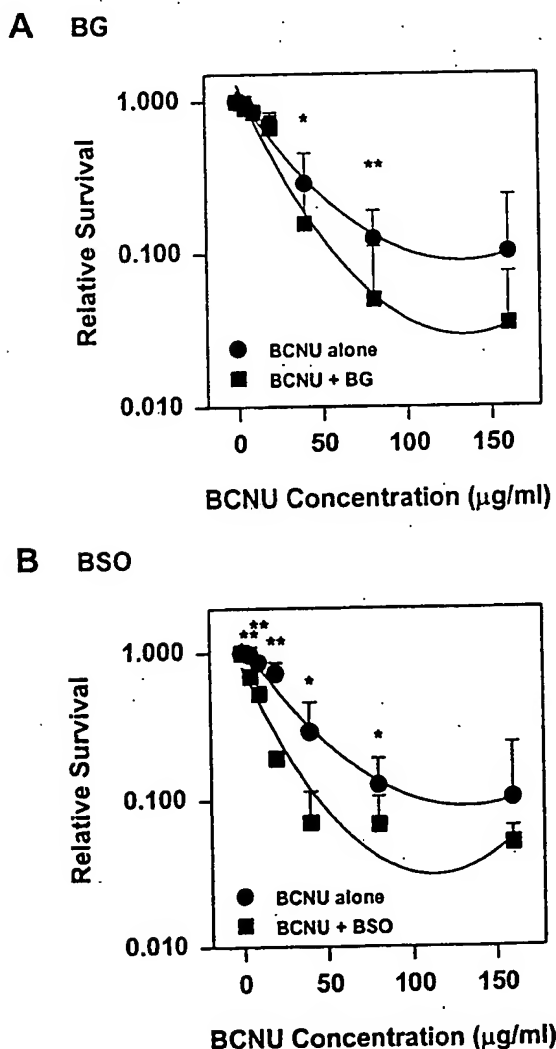


Fig. 3. Effect of BG and BSO pretreatment on the response of rat astrocytes to BCNU. Astrocytes were pretreated with either 25 μ M BG for 2 h (A) or 25 μ M BSO for 24 h (B) and then exposed to increasing concentrations of BCNU. Both BG and BSO pretreatment increased significantly the sensitivity of astrocytes to BCNU. Symbols represent the mean of at least two independent experiments, each performed in triplicate. \bullet , astrocytes treated with BCNU alone; \blacksquare , BG or BSO pretreatment. Bars, SE; asterisks, level of statistical significance (*, $P < 0.05$; **, $P < 0.005$).

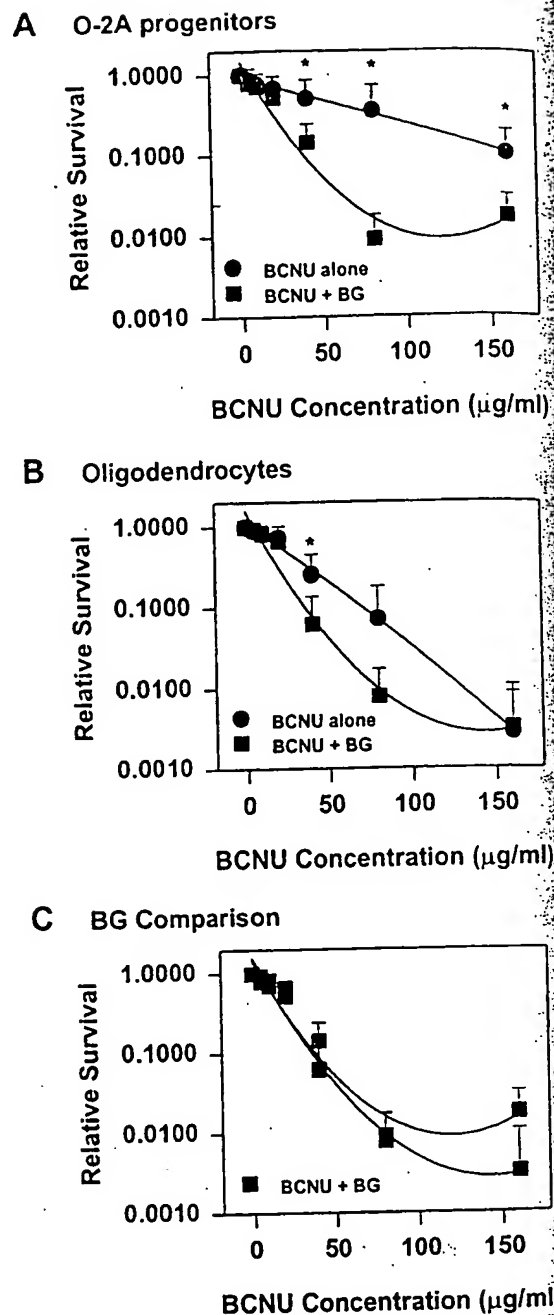


Fig. 4. Effect of BG pretreatment on the response of purified rat O-2A progenitor and oligodendrocytes to BCNU. O-2A progenitor cells (A) and oligodendrocytes (B) were pretreated with 25 μ M BG for 2 h and then exposed to increasing concentrations of BCNU. BG pretreatment appeared to increase the sensitivity to BCNU of both cell types. Interestingly, after negating the effects of *MGMT* on drug resistance by pretreatment with BG, O-2A progenitor cells and oligodendrocytes had similar sensitivities to BCNU. Symbols represent the means of at least two independent experiments, each performed in triplicate. \bullet , cells treated with BCNU alone; \blacksquare , BG pretreatment. Bars, SE; asterisks, level of statistical significance (*, $P < 0.05$).

treated oligodendrocytes. From these experiments, we conclude that *MGMT* contributes to the resistance of rat astrocytes to BCNU, but that other mechanisms of resistance also must be operative in these cells.

We suggest that a second component of BCNU resistance in astrocytes is related to levels of glutathione in these cells, which have elsewhere been shown to be higher than in O-2A progenitor cells (53). Modulation of glutathione has been demonstrated to alter resistance to a number of alkylating agents, including BCNU (29, 30, 32–33).

this reason, the effect of BSO pretreatment on the sensitivity of astrocytes was examined. BSO, an inhibitor of γ -glutamyl cysteine synthetase (the rate-limiting enzyme in glutathione biosynthesis), has been shown to cause depletion of cellular glutathione levels (30, 32-34, 48). BSO pretreatment increased the sensitivity of astrocytes to BCNU, suggesting that the glutathione-GST detoxification system, or some other aspect of glutathione physiology, may also play a role in the resistance of astrocytes to BCNU.

We also were surprised to find that sensitivity to BCNU treatment increases as O-2A progenitors differentiate into oligodendrocytes. Because MGMT levels were 5-fold higher in O-2A progenitor cells than in oligodendrocytes, we examined the effect of MGMT modulation on sensitivity to BCNU in purified cultures of O-2A progenitor cells and oligodendrocytes. BG pretreatment appeared to increase the sensitivity of both cell types to BCNU, although a greater increase in sensitivity was observed in the cultures of O-2A progenitors. These results suggested that MGMT contributes to BCNU resistance in cells of the oligodendrocyte lineage. Interestingly, the sensitivity of O-2A progenitors to BCNU after BG pretreatment is virtually indistinguishable from that of oligodendrocytes depleted of MGMT, suggesting that the down-regulation of MGMT is sufficient to account for the increased sensitivity of oligodendrocyte lineage cells to BCNU as they differentiate. If our general hypothesis that cellular lineage contributes to chemoresponsiveness is correct, then it will be of interest in future studies to determine whether the levels of MGMT in oligodendrogliomas or oligo-astrocytomas correlate with the extent to which maturation is precluded in these tumors.

In summary, we have demonstrated that rat glial cell subtypes exhibit differential expression of a number of drug resistance genes. In addition, it was determined that rat glial cell subtypes display a differential sensitivity to the alkylating agent BCNU. The increased resistance of astrocytes in comparison to oligodendrocytes was modulated, at least in part, by both BG and BSO, suggesting a role for both MGMT and glutathione in the resistance of astrocytes to BCNU. Thus, the sensitivity of normal rat glial cells to BCNU appeared to be dependent on more than one mechanism of drug resistance. Our experiments provide support for the hypothesis that the fundamental properties of glial cells may be retained in gliomas and may contribute to the differential chemosensitivity of glial neoplasms.

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